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Swapping of pro-sequences between keratinases of *Bacillus licheniformis* and *Bacillus pumilus*: Altered substrate specificity and thermostability

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ABSTRACT

Pro-sequences were swapped in *cis* between keratinases from *Bacillus licheniformis* (Ker BL) and *Bacillus pumilus* (Ker BP) to construct Ker ProBP–BL and Ker ProBL–BP, respectively. Expression of these keratinases was carried out constitutively by *E. coli* HB101-pEZZ18 system. They were characterized with respect to their parent enzymes, Ker BL and Ker BP, respectively. Ker ProBP–BL became more thermostable with a $t_{1/2}$ of 45 min at 80 °C contrary to Ker BL which was not stable beyond 60 °C. Similarly, the activity of Ker ProBP–BL on keratin and casein substrate, *i.e.* K:C ratio increased to 1.2 in comparison to 0.1 for Ker BL. Hydrolysis of insulin B-chain revealed that the cleavage sites increased to six from four in case of Ker ProBP–BL in comparison to Ker BL. However, cleavage sites decreased from seven to four in case of Ker ProBL–BP in comparison to the parent keratinase, Ker BP. Likewise, Ker ProBL–BP revealed altered pH and temperature kinetics with optima at pH 10 and 60 °C in comparison to Ker BP which had optima at pH 9 and 70 °C. It also cleaved soluble substrates with better efficiency in comparison to Ker BP with K:C ratio of 1.6. Pro-sequence mediated conformational changes were also observed in *trans* and were almost similar to the features acquired by the chimeras constructed in *cis* by swapping the pro-sequence region.

1. Introduction

Keratinases are subtilisin or metallo-proteases which have the capacity to act on recalcitrant proteins such as keratin. Their potential to act on β-keratin of chicken feather makes them prospective candidate for hydrolyzing proteinase-K resistant β-amyloid and prion plaques [1]. However, limited availability of efficient keratinases has boosted the search for better keratinases with improved catalytic efficiency for attacking recalcitrant proteins. At present, protein engineering techniques involving site-directed mutagenesis and DNA shuffling of core protein are used to develop better catalysts with improved substrate specificity and thermostability [2,3].

Proteases are expressed in pre pro form where pre-sequences are signal peptides responsible for mobilizing the proteases alongwith their pro-sequence across the cytoplasmic membrane. Pro-sequence region acts as chaperone leading active conformation in proteases in *Bacillus* [3]. Pro-sequences are subsequently autoprocessed or degraded by proteases to yield active protein into the extracellular medium [3]. Pro-sequences have been well recognized as hot spots for mutagenesis to develop conformational variants through pro-sequence engineering [3]. There are several reports where such variants have been developed by utilizing chaperone functions of an exogenous pro-sequence *i.e.* in *trans* or *in vitro* However, their use in *cis* exchanges where protease folding mediated by an exchanged pro-sequence, has been reported to result in functionally less active proteins [4].

Here, we report the effect of pro-sequence exchange on biochemical properties of keratinases from two closely related species, *Bacillus licheniformis* and *Bacillus pumilus*. The enzymes from these two species have been previously reported to possess contrasting features with respect to thermostability and substrate specificity [5,6].

2. Materials and methods

Restriction enzymes and DNA modifying enzymes were purchased from New England Biolabs (Beverly, USA). The oligonucleotides were synthesized by Sigma–Aldrich (USA). Genomic DNA extraction, plasmid extraction and gel elution kits were purchased from Qiagen, Hilden, Germany. Expression vector, pEZZ18 was purchased from GE Healthcare Science (India). *E. coli* HB101 was used as the expression host. All bacterial strains were grown on Luria-Bertani (LB) medium supplemented with 1% tryptone, 0.5% yeast extract, and 1% NaCl (pH 7.2). When required, ampicillin was added to the medium to a final concentration of 100 μg/mL.

Q-Sepharose, DEAE-Sepharose and various synthetic substrates were purchased from Sigma–Aldrich (USA).

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Table 1
Primer sequences used in cloning strategy of keratinases Ker ProBP–BL and Ker ProBL–BP.

Primer name	Sequence
Ker BPF	*Eco*RI 5′ *GAATTC*CACGGTCTCCAAAA 3′
Ker BPR	*Bam*HI 5′ *GGATCC*GTCATTCAATGC 3′
Ker BLF	*Sac*I 5′ *GAGCTC*CGCTCAACCGGCGAAAAAT 3′
Ker BLR	*Kpn*I 5′ *GGTACC*TTATTGAGCGGCAGCTTCGACA 3′

Italic bases stands for restriction sites.

2.1. In silico analysis of Ker BP and Ker BL

Pair-wise sequence alignment of Ker BL (GenBank accession no. AY590140) and Ker BP (GenBank accession no. HM219183) was done using the PSI-BLAST pre-profile processing (Homology-extended alignment) available from the PRALINE online resource portal (http://www.ibi.vu.nl/programs/pralinewww/) which uses an optimized heuristic with a gap opening penalty of 12 and an extension penalty of 1 [7,8]. The alignment was thereafter assessed based on their amino acid conservation and the motifs identified were analyzed with those already available in literature.

2.2. Cloning of Ker ProBP–BL and Ker ProBL–BP

Keratinases with swapped pro-sequences were created utilizing Ker BL and Ker BP clones which were already available in the laboratory in pEZZ18 vector [5,6]. pEZZ18-Ker BL was restricted with *Sac*I/*Kpn*I to obtain Ker BL and pEZZ18-Ker BP was restricted with *Eco*RI/*Bam*HI to obtain Ker BP. Restriction enzyme with single common restriction site of Ker BL and Ker BP gene was determined using the NEB cutter tool. *Psi*I restriction site was recognized in both Ker BL and Ker BP close to the pro-sequence region. Fallouts of Ker BL and Ker BP were thereby digested with *Psi*I and the digestion mixtures were mixed in a ratio of 1:1 to create a chimeric ligation mix. The ligation mix was then further ligated into *Sac*I/*Bam*HI and *Eco*RI/*Kpn*I digested pEZZ18 and transformed into *E. coli* HB101 to obtain chimeric pEZZ18-ProBP–BL (pro-sequence of Ker BP and mature protein from Ker BL) and pEZZ18-ProBL–BP (pro-sequence of Ker BL and mature protein from Ker BP), respectively. Positive clones were reconfirmed by colony PCR using a set of gene specific primer, Ker BPF-Ker BLR for Ker ProBP–BL and Ker BLF-Ker BPR for Ker ProBL–BP. The clones were subsequently sequenced at the Central Instrumentation Facility, University of Delhi. The primer sequences have been tabulated in Table 1 and the cloning strategy to form chimeric keratinases has been schematically presented in Fig. 1.

2.3. Expression of Ker ProBP–BL and Ker ProBL–BP

Extracellular expression of recombinant keratinases was carried out constitutively by *E. coli* HB 101-pEZZ18 system. The vector pEZZ18 has a *spa* promoter and protein A signal alongwith "ZZ" domain based on IgG binding sites [5]. Under the direction of protein A signal, the expressed protein gets secreted as a fusion protein with "ZZ" peptides under non-inducible condition [5]. *E. coli* HB101 cells harboring pEZZ18-Ker ProBP–BL and pEZZ18-Ker ProBL–BP were grown in LB medium supplemented with ampicillin at 37 °C, 300 rpm. After 18 h, the cells were separated by centrifugation at 7441 × *g* for 10 min and expression was checked in the extracellular broth by keratinase assay and SDS-PAGE analysis. Simultaneously, original keratinases Ker BL and Ker BP were also produced in the same manner [5,6].

2.4. Purification of Ker ProBP–BL and Ker ProBL–BP

The cell free culture broth was concentrated 10 times using ultrafiltration by 10 kDa molecular cut-off cassette. The retentate was applied to anion exchanger columns *i.e.* Q-Sepharose and DEAE-Sepharose pre-equilibrated with 10 mM Tris/HCl buffer, pH 8. The column was washed with the same buffer and 15 mL fraction was collected at a flow rate of 2 mL/min. Bound protein was eluted in a step gradient of sodium chloride (0.1–1 M NaCl). Purity of the protein was determined by SDS-PAGE analysis with the parent proteins *i.e.* Ker BP and Ker BL. Protein was concentrated with 50% (w/v) trichloroacetic acid (TCA) before loading onto the gel. The gel was stained with Coomassie brilliant blue R-250 solution.

2.5. N-terminal sequencing of Ker ProBP–BL and Ker ProBL–BP

After swapping the pro-sequences between keratinases, Ker BL and Ker BP their processing was confirmed by N-terminal analysis. The N-terminal sequence of the purified proteins, Ker ProBP–BL and Ker ProBL–BP was analyzed at the Protein Facility of Iowa State University, USA by automated Edman degradation performed with a 494 Procise Protein Sequencer/140C Analyzer (Applied Biosystems, Inc.).

2.5.1. Source of keratin substrate

Chicken feather was obtained from local poultry plants. They were washed thoroughly with triton X-100 (1%, w/v) and rinsed with distilled water followed by autoclaving at 15 psi. Thereafter, feather were dried in an oven at 60 °C for overnight and passed through a sieve of mesh no. 10 having pore size of 2 mm. Feather powder as a substrate was used to perform the keratinase assay.

2.5.2. Keratinase assay and protein estimation

Keratinase activity was measured as described by Dozie et al. [9] with some modifications. The assay mixture containing 1 mL of appropriately diluted enzyme, 4 mL of 50 mM glycine–NaOH buffer at optimum pH and 20 mg feather powder was incubated at optimum temperature for 1 h. The reaction was terminated by adding 4 mL of 5% (w/v) TCA and tubes were incubated at room temperature (25 ± 1 °C) for 1 h. Insoluble residues were removed by filtration through glass wool, and the filtrate was centrifuged at 7441 × *g* for 5 min. Control was set up by adding 20 mg feather powder, 1 mL of 5% trichloroacetic acid and 1 mL enzyme diluted in 3 mL of glycine–NaOH buffer. Proteolytic products in the supernatant were monitored at 280 nm. An increase in absorbance of 0.01 at 280 nm was considered as 1 U enzyme activity (1 KU = 1000 U).

The total protein was estimated by Bradford [10] taking bovine serum albumin (BSA) as the standard protein.

2.6. Biochemical characterization of Ker ProBP–BL and Ker ProBL–BP vs Ker BL and Ker BP

2.6.1. Effect of pH and temperature on activity and stability of Ker ProBP–BL and Ker ProBL–BP

The effect of pH was studied by performing the keratinase assay at different pH values ranging from pH 4 to 12 using 50 mM of each buffer including citrate phosphate (pH 4–6), sodium phosphate (pH 7), Tris–HCl (pH 8), glycine–NaOH (pH

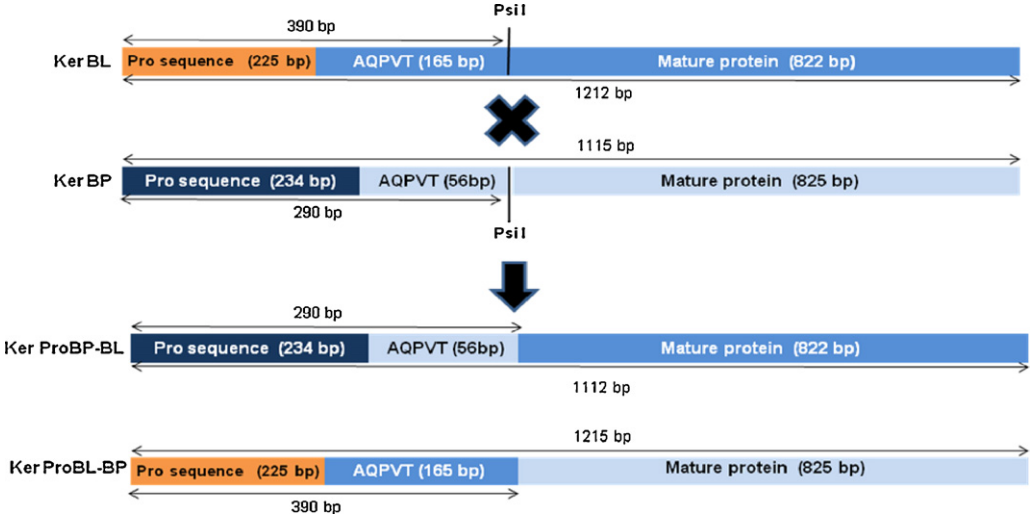

Fig. 1. Strategy for construction of keratinases Ker ProBP–BL and Ker ProBL–BP by swapping pro-sequence between keratinase from *B. licheniformis* (Ker BL) and *B. pumilus* (Ker BP) in *cis*.